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Breast cancer represents a major cause of death for women in the United States. Overexpression of HER-2/*neu* oncogene was found in approximately 30% of breast tumor tissues and shown to be a marker indicating poor prognosis for breast cancer patients. HER-2/*neu* overexpression in cancer cells is also known to enhance cancer metastasis and to induce chemoresistance to certain anti-cancer drugs and repression of HER-2/*neu* expression reduces malignancy of the cancer cells. Therefore, HER-2/*neu* overexpression serves as an excellent target for development of breast cancer therapy. Ribozymes have been successfully used to control gene expression. We have designed a novel suicide ribozyme that will allow a gene of interest (such as a toxin gene) to be expressed specifically in the HER-2/*neu*-overexpressing breast cancer cells, and therefore, will kill only the HER-2/*neu*-overexpressing cells. This report decribes the progress in the following specific aims: 1) Design of the suicide ribozyme and proof of concept *in vitro;* 2) Proof of concept *in vivo*: a reporter gene regulated by the suicide ribozyme will be expressed only in cells overexpressing HER-2/*neu* mRNA. 3) Application of concept *in vivo*: a toxin gene regulated by the suicide ribozyme will preferentially inhibit the growth of breast cancer cells that overexpress HER-2/*neu*. Success of this proposal will provide a novel way to target HER-2/*neu* overexpressing breast cancer cells and may improve treatment of breast cancer by gene therapy. In addition, the concept may be applied to control specific gene expression in other systems.

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INTRODUCTION

A. Background

1. Breast cancer is a major cause of death in the United States.

Data from the Surveillance, Epidemiology, and End Results (SEER) Program has indicated that breast cancer remains a major cause of death in this country (1, 2). It was estimated that more than 190,000 new cases of breast cancer would be diagnosed and approximately 50,000 women would die from this disease in the United States this year. It was also predicated that one in eight women will have breast cancer in their life span in the United States. **Therefore, there is an urgent need to develop novel anti-cancer agents to treat breast cancer.**

2. Amplification and overexpression of the HER-2/*neu* gene are frequently found in human breast cancer and other types of cancer

Amplification/overexpression of the HER-2/*neu* (also known as c-erb B2) gene was first found in approximately 30% of human breast cancer (3-8) . Several reports have revealed that the overall survival rate and time of relapse for those patients with HER-2/*neu* - overexpressing breast cancer is significantly shorter than those patients whose tumors do not overexpress HER-2/*neu*, indicating that HER-2/*neu* overexpression is an excellent prognostic marker (3, 8). **In addition to breast cancer, HER-2/*neu* overexpression has also been found in ovarian, lung, gastric, bladder, and oral cancers with high frequency (9-15).**

3. HER-2/*neu* overexpression provides an excellent target for cancer gene therapy

Gene therapy is an emerging field to develop novel strategies for cancer therapy. The P.I.'s laboratory has a long-standing interest in the role of HER-2/*neu* oncogene in cellular transformation, and has previously shown that a transcriptional repressor of HER-2/*neu* oncogene can function as a tumor suppressor for HER-2/*neu*-overexpressing cancer cells by delivering the gene encoding the transcriptional repressor with either

adenoviral or cationic liposome delivery system(16-18). These studies using animal models have revealed promising therapeutic effect (70%-80% of mice live longer than one year compared with control mice of which 100% died within a few months). However, both adenoviral and liposome delivery systems do not specifically target cancer cells, i.e. the tumor suppressor gene could be delivered into normal cells and potentially result in cytotoxicity. One way to overcome this problem is to design a specific delivery system for HER-2/*neu*-overexpressing cancer cells, such as immunoliposome that contains an antibody to target the HER-2/*neu*-encoded p185 surface protein (19). Alternatively, one may design an expression vector that will express a tumor suppressor gene or toxin gene only in the HER-2/*neu*-overexpressing cancer cells but not in normal cells. **We have proposed to target HER-2/*neu*-overexpressing breast cancer cells by designing a suicide ribozyme that will specifically express a toxin gene in cancer cells. Expression of the toxin gene is predicted to result in specific killing of cancer cells.**

4. Ribozyme can be used to control gene expression.

Ribozyme was originally discovered in small RNA pathogens (*Tetrahymena thermophilia)* of plants which can form a secondary structure resembling hammerhead and then self-cleave at the 3' side to the GUX triplet (where X can be C, A, or U) (20, 21). This secondary structure contains double-stranded RNA flanking sequences in which a sequence containing 5'GUX3' triplet should be located in one strand to serve as a substrate for cleavage (please see Fig.1 for a ribozyme structure). Artificially synthesized hammerhead RNA have been proven to behave like the natural ribozyme and can reduce the expression of the target RNA by cleavage of the target RNA sequence (22-24). Hence, ribozyme has become a useful technique to control gene expression by cleavage of target RNAs. However, direct application of the ribozyme (or antisense) approach to target an overexpressed RNA in cells is technically difficult as large number

of ribozyme (or antisense oligonucleotides or antisense RNA) molecules will be needed to block the expression of the overexpressed RNA. For example, in many HER-2/*neu*-overexpressing breast cancer cells, the overexpressed HER-2/*neu* mRNA is 100 fold more than that of normal breast epithelial cells (25). It will be much more difficult to directly block expression of HER-2/*neu* oncogene than that of other oncogenes which are expressed at lower levels in cancer cells using the ribozyme or antisense approaches. **We have proposed to take the advantage of the fact that an excess amount of HER-2/*neu* mRNA exists in the breast cancer cells and design a suicide ribozyme that will express a gene of interest only in the presence of overexpressed HER-2/*neu* mRNA. When this suicide ribozyme is fused to a gene encoding a toxin protein (or tumor suppressor), the toxin protein (or tumor suppressor) is predicted to be expressed only in the HER-2/*neu*-overexpressing breast cancer cells and kill the cancer cells.**

B. The purposes

The current proposal will develop a suicide ribozyme targeting at HER-2/*neu*-overexpressing breast cancer cells. The purposes are:

1. To design a suicide ribozyme structure and demonstrate the predicted ribozyme activity *in vitro*.

2. Proof of concept *in vivo*: to show that a reporter gene regulated by the suicide ribozyme is expressed only in breast cancer cell lines overexpressing HER-2/*neu* mRNA.

3. Application of concept *in vivo*: a toxin gene regulated by the suicide ribozyme will preferentially kill the breast cancer cells that overexpress HER-2/*neu* mRNA.

BODY

1. To design a suicide ribozyme structure and demonstrate the predicted ribozyme activity *in vitro*.

1.a. Design and working principle of the suicide ribozyme

The self-cleaving ribozyme, which is schematically shown in Fig. 1, contains 3 major portions : the suicide ribozyme, a therapeutic gene of interest, and a polyadenylation signal from 5' to 3' direction . The suicide ribozyme is consist of five domains: I, the 5' flanking binding sequence; II, the hammerhead catalytic domain sequence; III, the 3' flanking binding sequence; VI, the loop sequence and V, the substrate sequence from 5' to 3' direction. The two flanking binding sequences (I and III) are derived from the complementary strand of the human HER-2/ *neu* sequence so that they can form base pairs with the substrate sequence (part V) derived from human HER-2/*neu* mRNA. The catalytic domain (II) is chosen from the hammerhead ribozyme stem II which will catalyze the cleavage when the secondary structure showed in Fig. 1.A. is formed. The loop sequences (VI) are 5'AAAAAA 3' sequences which has been previously shown to facilitate the formation of ribozyme secondary structure (26, 27). The substrate sequence (V) is derived from the human HER-2/*neu* sequences which contains 5' GUX 3' in the middle serving as the cleavage site and other sequences forming base pairs with flanking binding sequences (I and III). Using RNA fold program to search for HER-2/*neu* gene sequences that fulfill the requirement for the cleavage sequence V, we have identified 40 regions that may be used as the substrate sequences. The following encouraging results shown in Fig. 2 and 3 were obtained using 5' CCCAGUGU**GUC**AACUGCAGCC 3' [corresponding to nucleotide 1750 to 1771 of human HER-2/*neu* (5)] as the substrate sequence V and its complementary portion as flanking binding sequence I and III. In the absence of exogenous HER-2/*neu* mRNA, the suicide ribozyme will form the secondary

structure as shown in Fig. 1. A, and the cleavage will occur. As a consequence, the polyadenylation signal that is critical for mRNA stability will no longer be covalently linked to the mRNA of the fused gene containing both therapeutic gene and the suicide ribozyme sequence and the mRNA will become unstable and be quickly degraded. Thus, the protein product of the therapeutic gene cannot be expressed. On the other hand, when a large amount of cellular HER-2/*neu* mRNA exists, it will compete with the substrate sequence (V) to hybridize with the flanking binding sequence (I and III) and a structure depicted in Fig. 1.B. may form. This structure will cleave the cellular HER-2/*neu* mRNA and preserve the polyadenylation sequence. Thus the therapeutic protein can be expressed in an environment containing excess of HER-2/*neu* mRNA.

1.b. Proof of concept using *in vitro* transcribed RNA.

To examine whether the ribozyme designed in Fig. 1 can self-cleave and the cellular HER-2/*neu* RNA can prevent self-cleavage as proposed, we placed the suicide ribozyme sequence (I to V in Fig. 1. A.) in an i*n vitro* transcription vector driven by T7 bacterial promoter. When the transcript is synthesized by *in vitro* transcription system (Stratagene, La Jolla, CA), we expect to detect two products with 14 nt and 90 nt in length if self-cleavage occurs. As shown in Fig. 2, the full length transcript (104 nt) was readily detected by 2 minutes after *in vitro* transcription was started (At zero minute no transcript was detected, data not shown). However , a small amount of cleaved product, P1(90 nt) was also detected, indicating that self-cleavage occurred within a very short time after full length transcript was made. While the reaction proceeded, the amount of transcripts were increased as evident from the enhanced intensities from both full length transcript and cleaved products. However, the ratio between the full length transcript and cleaved product (e.g. P1) was decreased. By 16 minutes, approximately 70% of the transcripts were cleaved products (please compare the relative intensity between full length transcript and P1). The P2 product contains only 14 nt and therefore its intensity

(which is proportional to the length of transcript) was too weak to be quantitated. The results demonstrated that the designed ribozyme is associated with high efficiency of self-cleavage. To test whether the self-cleavage activity can be inhibited by HER-2/*neu* RNA, exogenous HER-2/*neu* RNA was added to the *in vitro* transcription system. As shown in Fig. 3, in the presence of exogenous HER-2/*neu* RNA the intensity of the full length transcript was significantly increased (Fig. 3, lanes 2 and 4 compared with lanes 1 and 3), suggesting inhibition of the self-cleavage activity. A better separation between the full length transcript and P1 product in Fig. 3 than in Fig. 2 is due to the difference in the density of polyacrylamide gel (20% Vs 10%, Please see figure legend). These results provide strong evidence that the proposed concept works i*n vitro.*

2. Proof of concept *in vivo.*

Based on the encouraging data shown in Figs. 2-3, we further constructed an expression vector using the pcDNAI vector (Invitrogene, San Diego, CA) that contains CMV promoter and polyadenylation signal from SV40 virus. We placed the ribozyme sequences 3' to the reporter gene encoding chloramphenicol acetyltransferase (CAT) expecting that self-cleavage of the ribozyme would make the CAT-containing mRNA unstable and quickly degraded, therefore, no or very weak CAT activity can be detected. However, in the presence of HER-2/*neu* mRNA, the HER-2/*neu* mRNA will be complementary to the flanking binding sequences and prevent formation of self-cleavage ribozyme. Thus, the CAT-encoding mRNA will be able to produce CAT protein that can be easily detected by enzymatic activity of chloramphenical acetyltransferase. We have compared the CAT expression efficacy between two human breast cancer cell lines, the HER-2/*neu*-overexpressing MDA-MB-453 cells and the HER-2/*neu* low-expressor, MDA-MB-231 by transfecting the CAT-suicide ribozyme constructs into these cells. The transfection efficiency was normalized by ß-gal activity that indicated similar transfection efficiency between these two cell lines (RSV-LacZ plasmid was co-transfected with the

CAT-suicide ribozyme construct to provide ß-gal activity for normalization of transfection efficiency). As shown in Fig. 4, we have observed higher CAT activity in MDA-MB-453 cells then in MDA-MB-231 cells. However, when we carried out similar experiments in other breast cancer cell lines such as the HER-2/*neu*-overexpressing MDA-MB-361, BT-474 and SkBr3 cells, and the HER-2/*neu* low-expressing MDA-MB-435, MCF-7 cells, we did not observe higher CAT activity in the HER-2/*neu* overexpressors than in the low-expressors as we originally expected. Since these breast cancer cell lines were established from different breast cancer patients, they may have other genetic differences in additional to the different HER-2/*neu* expression. To confirm that the differential expression of the CAT-suicide ribozyme construct is due to difference in HER-2/*neu* mRNA expression and not due to other genetic changes in these cells, we will co-transfect the CAT-suicide ribozyme construct and human HER-2/*neu* expression vector (p-CMV-HER-2) into a HER-2/*neu* low-expressor, such as MCF-7. The p-CMV-HER-2 will express HER-2/*neu* mRNA and therefore may enhance CAT activity when comparing with the control experiment that will be co-transfected with the CAT-suicide ribozyme and a pCMV vector which dose not contain the HER-2/*neu* gene. Using the reporter CAT gene, we should be able to establish an expression vector that will express preferentially in the presence of excess of HER-2/*neu* mRNA.

3. Application of concept *in vivo*.

We will test whether a HER-2/*neu* overexpression-dependent expression vector can be applied to kill the HER-2/*neu*-overexpressing breast cancer cells. The CAT gene will be replaced by a toxin gene, a gene encoding diphtheria toxin A-chain (DT-A) toxin. Expression of the toxin protein in cells will kill the cells by inhibiting protein synthesis (28). Cell killing can be measured by simply counting the number of survival cells. We already obtained the DT-A expression vector that contains both DT-A gene and a selection marker, neomycin-resistant gene. This toxin-ribozyme construct will be

transfected into HER-2/*neu* overexpressors and low-expressors, respectively. Since it contains the neomycin-resistant gene which will enable the transfected cells to be resistant to the antibiotics G418. Transfectants will be selected by G418 and the G418-resistant colonies will be counted. In the HER-2/*neu* -overexpressing breast cancer cells, excess of HER-2/*neu* mRNA will prevent the suicide ribozyme from self-cleaving and therefore allow expression of the toxin gene, which will eventually kill the cells. We expect the number of G418-resistant colonies to be dramatically reduced compared with the control experiments in which the vector containing only the neomycin-resistant gene but not the toxin gene is transfected into the cells. In the HER-2/*neu* low-expressors, the toxin gene can not be expressed (or expressed at a much lower level) because the self-cleavage of the suicide ribozyme will destabilize the toxin mRNA. Therefore, the number of G418-resistant colonies will be similar to (or slightly less than) that produced by transfection with the neomycin-resistant gene alone. The proposal is based on a simple but novel concept to express a therapeutic protein. One critical point to apply this concept successfully is the balance between self-cleavage and the inhibition of self-cleavage. Low efficiency of self-cleavage will generate leaky activity and insufficient inhibition of self-cleavage may not produce enough stable mRNA to express the therapeutic protein. This issue is currently carefully addressed by changing the length of double-stranded RNA between the flanking binding sequences (I and III) and the substrate sequences (V).

4. An alternative approach using iron responsive element to control selective expression in HER2/neu-overexpressing cancer cells

In addition to the ribozyme approach described above, we are currently testing the possibility of targeting HER2-overexpressing cells based on another novel approach which utilizes a translation regulator, the iron responsive element (IRE), to preferentially express the luciferase reporter gene in the HER-2/neu overexpressing breast cancer cell lines. IRE is a stem-loop RNA structure located in the 5' non-coding region of the ferritin mRNA, and was first identified as a negative translation regulator when it binds to a specific cytoplasmic protein (IRP) at low iron level (29). When the intracellular level of iron is raised the IRP is dissociated from the IRE resulting in the activation of ferritin mRNA by becoming associated with polysomes. By base-pairing to a antisense sequence, the IRE stem-loop structure will be abolished and allowing the mRNA to be translated (Figure 5B). A short HER-2/neu coding sequence that is antisense to a consensus sequence of the iron responsive element (IRE) has been identified (Figure 5A). To test whether this IRE-antisense sequence can abolish the authentic IRE stem loop and facilitate expression of the controlled reporter gene, we have generated a construct that contains a consensus IRE at the 5' untranslated region (5'UTR) of the luciferase gene driven by a cytomegalovirus (CMV) enhancer-promoter (IRE-LUC) (Figure 5C). This particular IRE possesses a twenty-two nucleotides antisense sequence to a region of the human HER-2/neu coding sequence (between +800 and +821) (Figure 5B) (30). As a control, a CMV-driven luciferase gene was also constructed without an IRE (LUC) (Figure 5C). These two constructs along with an internal control CMV-lacZ gene were transfected into cells by electroporation. The luciferase activity was then normalized by the β-galactosidase activity.

The breast cancer cell lines, MDA-MB-435, which expresses a basal level of HER-2/neu mRNA, and MDA-MB-435eb1, which is a stable transfectant of MDA-MB-435 cells

with HER-2/neu gene and expresses a high level of HER-2/neu mRNA, were used to test our hypothesis. As shown in Figure 6A, the luciferase activity of IRE-LUC is 38- and 14-fold less than LUC in MDA-MB-435 and MDA-MB-435 eb1, respectively. This result indicates that the presence of this particular IRE could greatly reduce the luciferase expression as compared with the one without IRE. This observation is consistent with the translation inhibitory function of a regular IRE. While there is no significant difference between these two cell lines in the expression of LUC, an approximately 3-fold higher luciferase activity of IRE-LUC was observed in the HER-2/neu overexpressing MDA-MB-435eb1 cells than in its parental cell line, MDA-MB-435 (Figure 6B). Thus, the preferential expression of IRE-LUC may be contributed by the overexpressed HER-2/neu mRNA, presumably by forming a sense-antisense hybrid with this IRE and abolish its translation inhibitory function. To further demonstrate the utility of this approach to selectively target the HER-2/neu overexpressing cells, we chose several human breast cancer cell lines which are either expressing basal levels of HER-2/neu mRNA, i.e. HBL-100, MDA-MB-435, and MDA-MB-468, or a high level of HER-2/neu, i.e. MDA-MB-453, and AU565, as recipients of the test constructs. The LUC construct showed at least 30-fold higher expression than that of IRE-LUC in these cell lines (data not shown). More importantly, as shown in Figure 7, the relative expression of IRE-LUC is at least 6-fold higher in the high expressors, MDA-MB-453 and AU565, than that in a low expressor, HBL-100; about 4-fold higher than in MDA-MB-435; and about 5-fold higher than in MDA-MB-468.

The design of the antisense IRE is relatively easy since the IRE consensus sequence allows some flexibility in the length of the "lower" stem and in the variability of nucleotide sequence on both "upper" and "lower" stems (Figure 5A). It needs to be tested to see if optimal structures would confer better selectivity of expression than the region reported in this study. For example, although the number of base pairs of the "upper" stem is fixed at five, the "lower" stem, however, could tolerate variable length.

By increasing the length of the "lower" stem, one could presumably achieve better targeting efficiency with higher sequence specificity. It is obvious that once the optimal IRE was obtained in vitro, then it could be placed 5' to a therapeutic gene, such as a toxin, and test the construct in animals to determine its efficacy of delivery and the degree of selective killing of tumor cells overexpressing a given gene in vivo. Similar to the suicide ribozyme approach described earlier, this approach may provide another way to selectively express a toxin gene in HER2/neu-overexpressing cancer cells. It is too early to determine which method will work more efficiently. We will continue to work on both approaches.

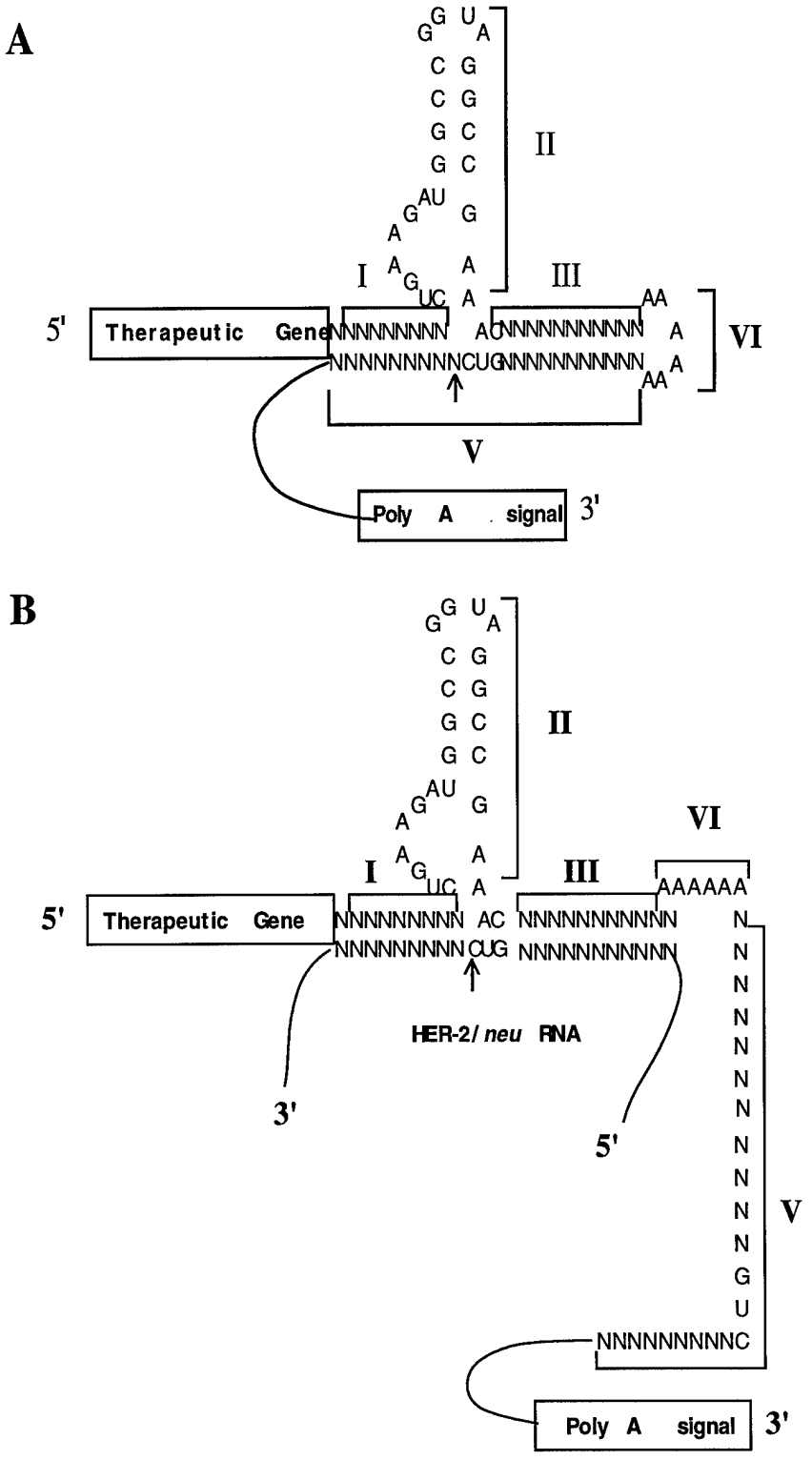

Fig 1. Schematic of suicide ribozyme design. Parts I-V are defined in the text . The arrowheads indicate the cleavage site. Panel A represents a predicted suicide ribozyme structure. Panel B shows a secondary structure between the ribozyme and HER-2/*neu* RNA . This structure will block the self cleavage of suicide ribozyme.

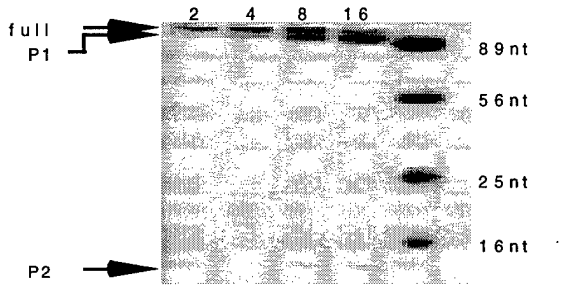

Fig 2. *In vitro* self-cleavage of suicide ribozyme. The suicide ribozyme was *in vitro* transcribed using ^{32} P-UTP as labeling. At different time point (2, 4, 8,16 min), the products were stopped by stop buffer and loaded on the 20% 7M urea denatured polyacrymide gel. The self-cleavage was visualized by exposure of the gel to X-ray film. The amount of full length transcripts (Full) were reduced and the cleaved products (P1 and P2) were increased with increasing of the reaction time.

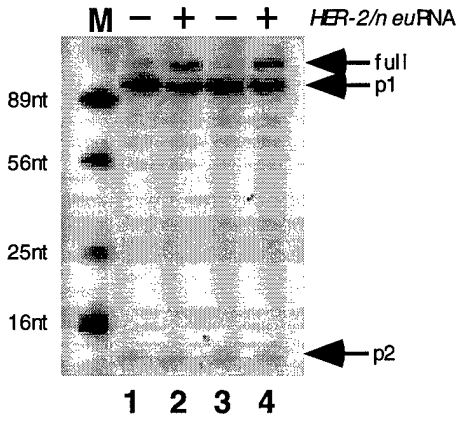

Fig 3. *In vitro* blockage of suicide ribozyme self-cleavage by *HER-2/neu* RNA. The exogenous *HER-2/neu* RNAs were added into the reaction mixture containing 0.5 μg template DNA encoding ribozyme before *in vitro* transcription. 30 min later, the reaction were stopped by stop buffer. The results were analyzed using 10% 7M urea denatured polyacrymide gel. The materials added to the reaction mixture are: **1.** nonspecific RNA derived from *in vitro* transcription. **2.** *HER-2/neu in vitro* transcripts (*HER-2/neu* : ribozyme 50:1). **3.** 200ng total RNA from the *HER-2/neu* low-expressing cells, MDA-MB-231, or **4.** 200ng total RNA from the *HER/neu*-overexpressing cancer cells, MDA-MB-453. Full: full length suicide ribozyme. P1 and P2: cleavage products. The molecular size standards were indicated on the left.

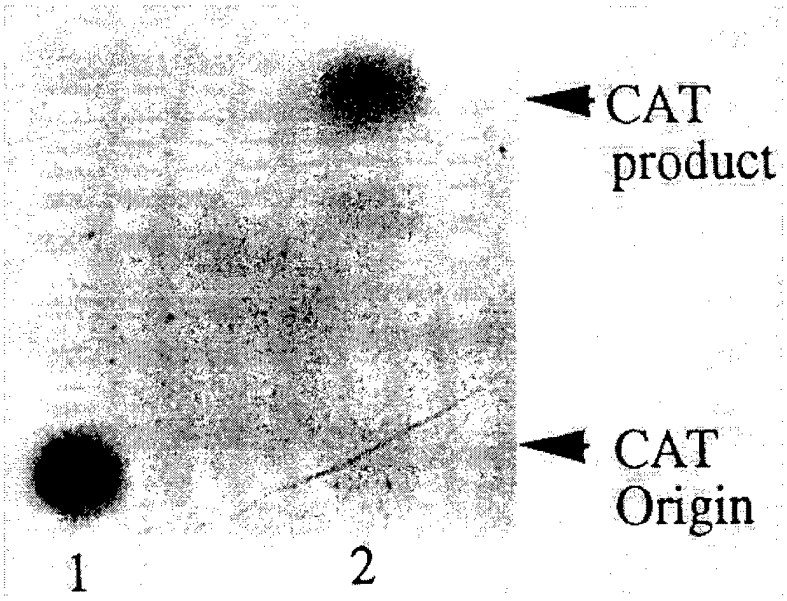

Fig 4. Preferential expression of CAT reporter gene in *HER-2/neu*-overexpressing cancer cells by CAT-suicide ribozyme. 1 μg CAT-Suicide ribozyme were cotransfected with 4 μg RSV-LacZ into human breast cancer cell lines with overexpressed *HER-2/neu* (MDA-MB-453) or low-expressed *HER-2/neu* (MDA-MB-231). The total 15 μg DNA was adjusted by the pGEM3 plasmid. Forty eight hours later, the cells were harvested. The cell extracts normalized by β-gal assay were applied to CAT reaction. Four hours later, the reaction products were loaded on to a TLC plate. Lane **1.** *HER-2/neu* low expressor, MDA-MB-231. Lane **2.** *HER-2/neu* overexpressor, MDA-MB-453.

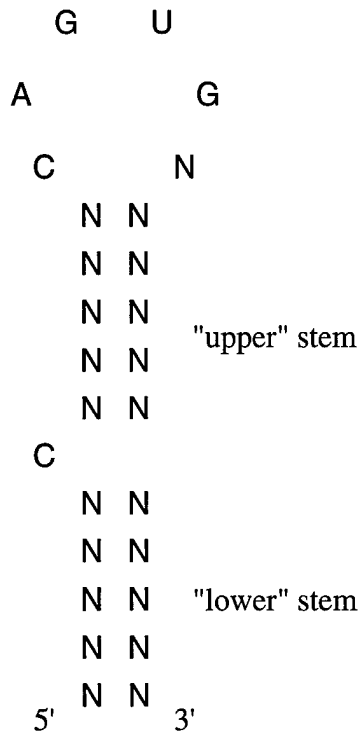

Figure 5A The IRE consensus sequence and stem-loop structure. A six-member loop, the first five bases are usually CAGUG and the sixth base is often a pyrimidine. The "upper" stem usually consists of five base pairs with any complementary nucleotides. A "C" bulge is invariable. The "lower" stem is of various length with any complementary nucleotides but it would also tolerate some unpaired bases.

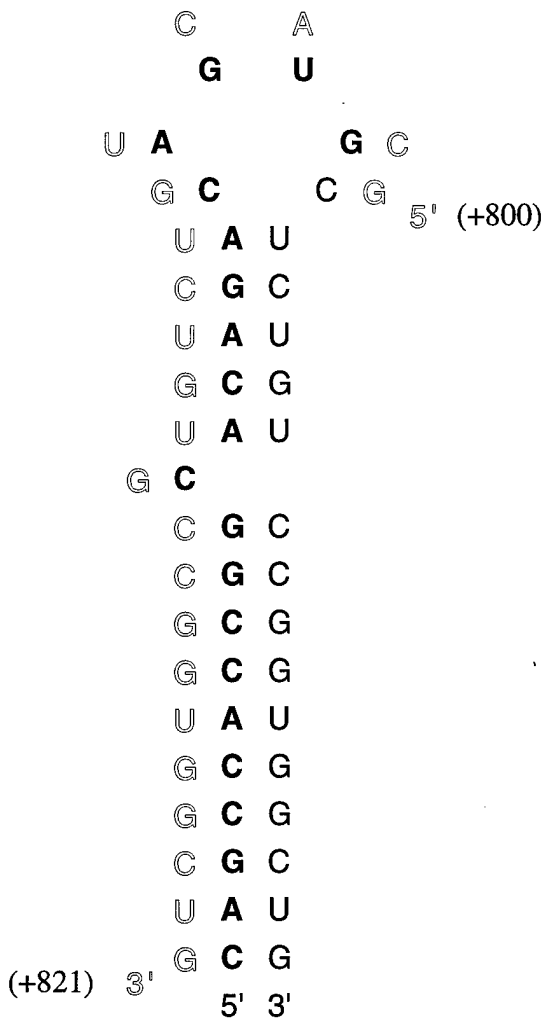

Figure 5B The design of an anti-sense HER-2/neu IRE sequence. The anti-sense sequence to a HER-2/neu coding region is shown in bold face letters. The coding region of HER-2/neu (from +800 to +821) is shown in outlined letters.

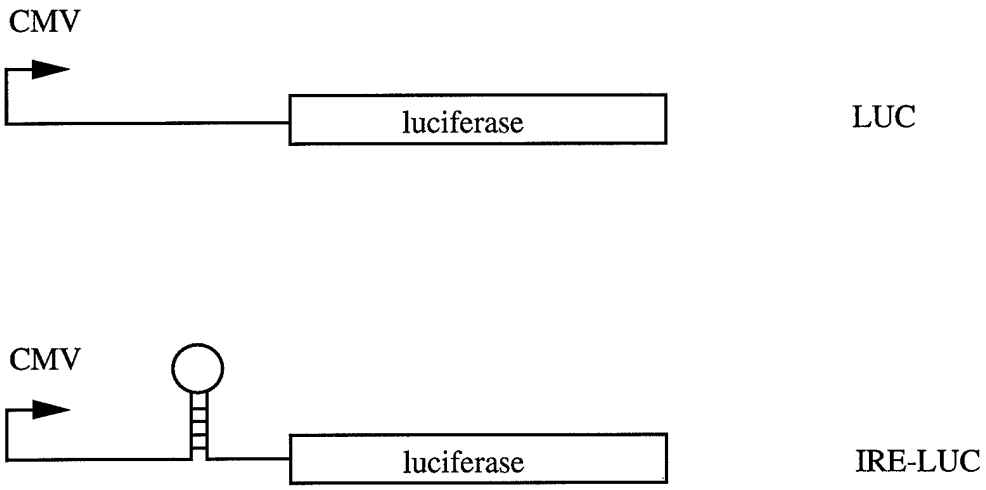

Figure 5C The mRNAs of the luciferase gene driven by CMV promoter either without (LUC) or with (IRE-LUC) IRE located at the 5' UTR.

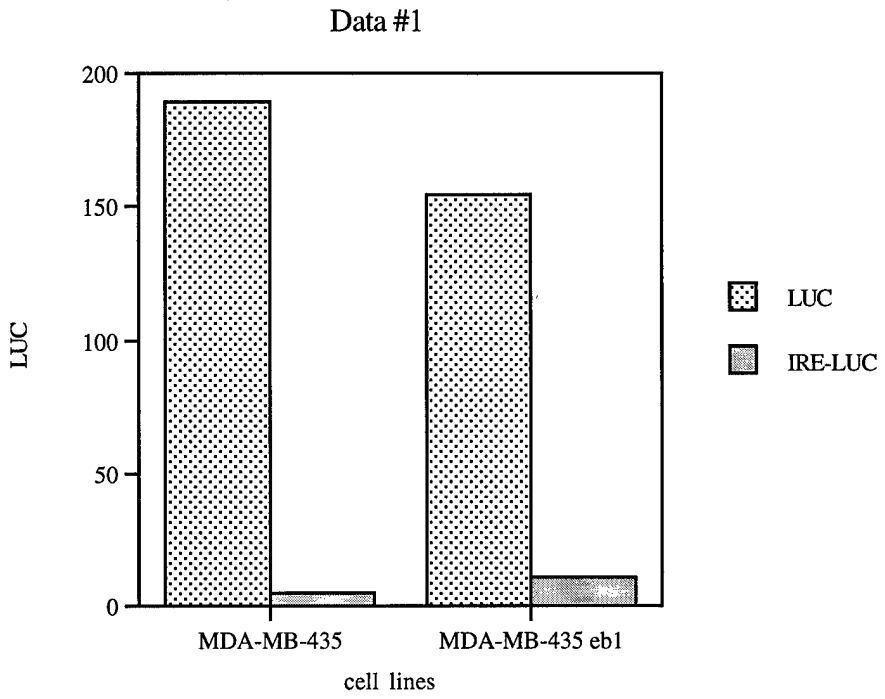

Figure 6A Luciferase activity with or without IRE in MDA-MB-435 and MDA-MB-435eb1. Five micrograms of either LUC or IRE-LUC and two micrograms of CMV-lacZ were electroporated into MDA-MB-435 and MDA-MB-435 eb1 cells. The luciferase activity was assayed 21-24 h after transfection and the adjusted luciferase activity was normalized by the b-galactosidase activity to standardize the transfection efficiency.

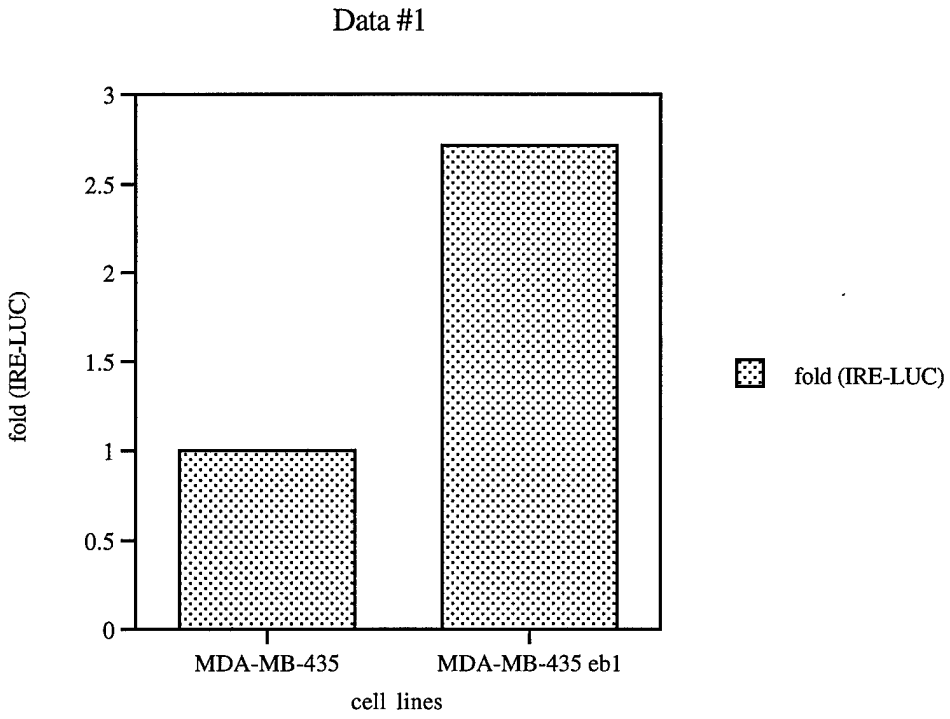

Figure 6B. The fold difference between the luciferase activity of IRE-LUC in MDA-MB-435 and MDA-MB-435 eb1. It was obtained by normalizing both the b-galactosidase activity and the luciferase activity of LUC in both cell lines and set the corrected luciferase activity in MDA-MB-435 as one.

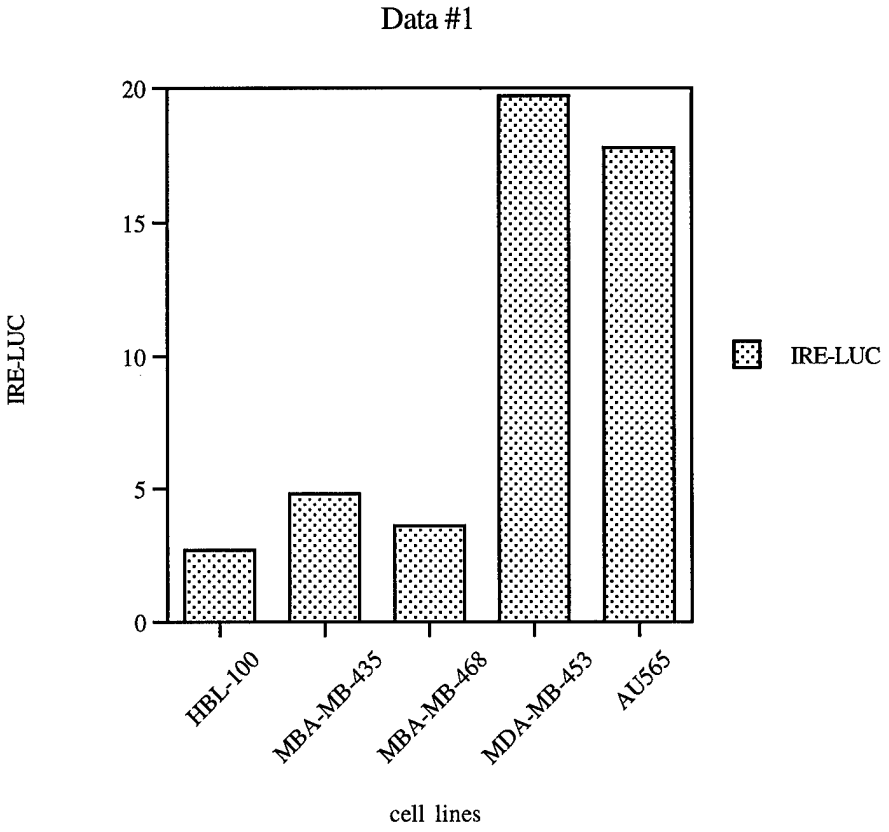

Figure 7. The luciferase activity of IRE-LUC in human breast cancer cell lines. Five micrograms of IRE-LUC anmd two micrograms of CMV-lacZ were electroporated into several human breast cancer cell lines which either express endogenous level of HER-2/neu mRNA, HBL-100, MDA-MB-435, and MDA-MB-468, or high expressors, MDA-MB-453 and AU565. The luciferase activities have been normalized by the b-galactosidase activities to equalize the transfection efficiency.

CONCLUSION

Task 1: Design of optimal suicide ribozymes

a. In addition to the suicide ribozyme described in the Specific Aim 1, we are currently synthesize other constructs that use other regions of the HER-2/neu sequences that also meet the requirement for ribozyme secondary structure to find a optimal suicide ribozyme for the HER-2/neu-overexpressing breast cancer cells.

b. We are also in the process of changing the length of cleavage sequence V and its complementary flanking binding sequences (I and III) to find an optimal structure for the suicide ribozyme.

Task 2: Examination of efficiency of suicide ribozymes *in vitro*

The efficiency of one suicide ribozyme has been tested as shown in Figure 2 and Figure 3.

Task 3: Application of concept *in vivo*

It is not yet clear why the data shown in Figure 4 can only be shown for the MDA-MB-453 and MDA-MB-231 breast cancer cells. And when other cells were used, no preferential expression in the HER-2/neu-overexpressing cancer cells such as MDA-MB-361, BT-474, and SKBr3 can be demonstrated. We therefore need to modify the length of cleavage sequence V and its complementary flanking binding sequence or synthesize other constructs. In addition, we have also developed another approach to design a vector for preferential expression in the HER-2/neu-overexpressing cancer cells. As shown in Figures 5-7, this alternative approach using iron response element may also provide a way to reach our goal for the development of a vector preferentially expressing a gene in

the HER-2/neu-overexpressing breast cancer cells. We will continue to optimize both approaches to obtain a better vector to reach our goal.

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